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Endogenously produced adenosine regulates articular cartilage matrix homeostasis: enzymatic depletion of adenosine stimulates matrix degradation

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Summary

Objective: Enhanced extracellular levels of adenosine have been shown to inhibit experimentally induced cartilage degradation. The objective of this study was to investigate the role of adenosine and A_2 adenosine receptors in regulating cartilage homeostasis in the absence of inflammatory stimuli.

Methods: Cartilage explants were exposed to adenosine deaminase (ADA) to deplete extracellular adenosine, and conditioned medium was collected for evaluation of glycosaminoglycan (GAG), prostaglandin E_2 (PGE_2), nitric oxide (NO), and matrix metalloproteinases-3 and -13 (MMP-3, MMP-13) levels. In a second set of experiments, cartilage incubated with ADA was simultaneously exposed to the adenosine kinase inhibitor 5′-iodotubercidin (ITU) to inhibit adenosine breakdown, or to the A_{2A} adenosine receptor agonist N^6-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA). Finally, explants were incubated with the adenosine receptor antagonists ZM241385, CGS15943, theophylline or caffeine to block normal receptor activation by endogenous adenosine.

Results: Exposure to ADA induced a concentration-dependent increase in GAG release and production of total MMP-3, MMP-13, PGE_2, and NO. Both ITU and DPMA inhibited the ADA-mediated increases in GAG release and PGE_2, and NO production, but only ITU inhibited MMP-13 release. Exposure to ZM 241385 increased GAG, MMP-3 and MMP-13 release. Additionally, CGS 15943 increased MMP-3 production while theophylline increased GAG, PGE_2, and NO release.

Conclusions: Endogenous adenosine levels appear to regulate cartilage matrix homeostasis even in the absence of inflammation. Regulation occurs, at least in part, through activation of cell surface receptors. This study suggests that autocrine and paracrine responses to adenosine release are important for maintenance of healthy articular cartilage.

Key words: Adenosine, Adenosine Deaminase, Cartilage, Degradation.

Introduction

The purine base adenosine is a naturally occurring multifunctional signaling molecule capable of eliciting anti-inflammatory responses in several *in vitro* and *in vivo* models of inflammation[1–5]. The therapeutic potential of adenosine for managing inflammatory joint disease has been investigated in previous studies documenting its ability to limit synoviocyte[6] and chondrocyte[7,8] inflammatory responses and to minimize articular damage in adjuvant-induced models of arthritis in rats[9–11]. Although the physiological effects of adenosine stimulation have been studied in the presence of naturally occurring and experimentally induced inflammation, the role of adenosine as an autocrine or paracrine regulator of chondrocytes and cartilage matrix homeostasis has not been investigated previously.

Extracellular adenosine activates P1 purinergic cell surface receptors that have been categorized into four subclasses: the A_1 and A_3 receptor subclasses that are negatively coupled to adenylate cyclase and the A_{2A} and A_{2B} that stimulate adenylate cyclase and increase cAMP production[12]. Chondrocytes in articular cartilage are sparsely distributed in a dense extracellular matrix and must rely on diffusible signals in lieu of direct cell to cell contact to communicate with one another. Recognizing the fact that chondrocytes are capable of releasing adenosine[13], we hypothesized that local adenosine release from chondrocytes could provide an important intra-articular signal regulating cartilage homeostasis.

The availability of adenosine as an endogenous extracellular signaling molecule would be regulated by its short half-life, typically less than one minute in biological systems[13–16]. Rapid phosphorylation of adenosine to form

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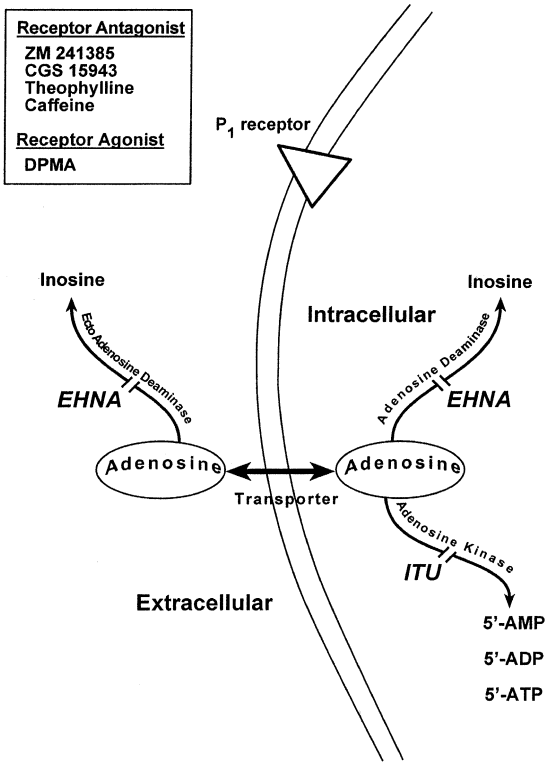

Fig. 1. Adenosine metabolism and cell surface receptor subclasses. Extracellular adenosine can be deaminated to inosine by ADA or can be transported into the cell and degraded by ADA or adenosine kinase.

ATP occurs within the cell through the actions of adenosine kinase[17]. Alternatively, in the extracellular space and cytosol, adenosine is metabolized by enzymes including adenosine deaminase (ADA), which irreversibly converts adenosine to inosine (Fig. 1). Adenosine deaminase 1 is a predominately cellular form while ADA 2 is more prevalent in serum[18–20]. Elevated levels of both forms of ADA have been reported in patients suffering from rheumatoid arthritis[21–23]. Those studies suggest that a reduction in local levels of adenosine may contribute to the synovial inflammation associated with naturally occurring arthritic conditions including immune mediated arthritis. The amount of adenosine available to stimulate cell surface receptors is dependent upon the balance between the release, uptake and hydrolysis of extracellular adenosine[24]. Fluctuations in the pathways that produce or consume adenosine could significantly impact extracellular adenosine concentrations and alter receptor stimulation.

The purpose of the study reported here was to investigate the role of endogenous adenosine in maintaining cartilage matrix homeostasis. This was first evaluated by enzymatically depleting extracellular adenosine through the addition of ADA. We then investigated whether an adenosine kinase inhibitor, that increases local adenosine levels, could counter the effects of ADA supplementation. In addition, we stimulated adenosine receptors with an A_{2A} adenosine receptor agonist to determine if we could block the effects of ADA exposure through receptor activation. Finally, we used adenosine receptor antagonists to determine whether we could mimic the effects of ADA exposure by blocking cell surface adenosine receptor activation. The physiological impact of altered adenosine signaling was evaluated by measuring glycosaminoglycan (GAG) release, prostaglandin E_2 (PGE_2) synthesis, nitric oxide (NO) production, and matrix metalloproteinase (MMP)-3 and MMP-13 levels.

Materials and methods

MATERIALS

Commercial immunoassays for PGE_2 and MMP-3 were purchased from R&D Systems (Minneapolis, MN, U.S.A.) while those for MMP-13 were from Amersham Biosciences (Piscataway, U.S.A.). All standard chemicals and reagents including ADA 1, DPMA, ITU, caffeine, theophylline and CGS 15943 were obtained from Sigma-RBI (St. Louis, MO, U.S.A.) while ZM 241385 was purchased from Tocris (Ellisville, MO, U.S.A). Tissue culture medium, fetal calf serum (FCS) and amphotericin B were purchased from Gibco-BRL (Grand Island, NY, U.S.A.), while gentamicin sulfate was obtained from Schering-Plough Animal Health Corp (Madison, NJ, U.S.A.).

TISSUE COLLECTION

Full-thickness articular cartilage was dissected from the metacarpophalangeal and metatarsophalangeal joints of fresh equine cadavers, as previously described[25]. Cartilage was harvested from 18 horses between the ages of 1 and 8 years that died or were euthanized for problems unrelated to the musculoskeletal system. At the time of tissue collection, all joints appeared normal with no evidence of synovial effusion or synovial proliferation, and the articular cartilage was also grossly normal. Cartilage was collected under aseptic conditions into Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM-F12) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 50 µg/ml gentamicin sulfate, and 50 µg/ml ascorbate. Tissue was rinsed and transferred to fresh DMEM-F12 medium containing the same supplements as well as 5% FCS. In this report, the term control medium refers to fresh DMEM-F12 medium with the previously mentioned supplements and 5% FCS. Cartilage was incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

MONOLAYER VIABILITY ASSAY

Chondrocytes were freed from the extracellular matrix by use of 3-step enzymatic digestion, as previously described[25]. Following digestion, undigested matrix was removed by passing the digest through a 70-µm cell strainer. Released chondrocytes were collected by centrifugation at 230 ***g*** for 6 min. Freshly isolated chondrocytes were plated in 24-well plates at a density of 5×10^5 cells/cm^2. Plated chondrocytes were allowed to stabilize in control medium for 24 h, and confluence was confirmed prior to initiating experimental incubations. Treated chondrocytes were then incubated for 24 h with ADA (0.5 to 2 U/ml). Following ADA exposure, the medium was discarded and cells were incubated in the dark for 1 h at 37°C with 0.5 ml of 0.6 mg/ml of methyl-tetrazolium dye[26]. After incubation, the dye was aspirated and cells were solubilized by the addition of 2 ml of dimethyl sulfoxide per well. The contents of each well were then mixed thoroughly with 0.675 ml of Sorensen buffer (0.1 M glycine, 0.1 M NaCl [pH 10.5]). Finally, 200 µl aliquots from each well were

transferred to a clear 96-well plate and the optical density was measured at 570 nm using a microplate reader. All samples and standards were assayed in triplicate.

EXPLANT CULTURE

At 24 h post-collection, 10 to 15 mm^3 cartilage explants were cut from harvested cartilage. Explants were weighed and combined to a total wet weight of 80 to 105 mg per replicate, and each individual replicate was stored in a sterile polystyrene tube in 2 ml of fresh control media. Explants were allowed to stabilize for 36 to 48 h prior to the initiation of experimental protocols.

Cartilage was not pooled between horses, and individual experiments used cartilage collected from a single horse to allow within animal comparisons. Cartilage from three to eight different horses was used to evaluate the effects of each reagent on matrix metabolism. Explants from individual horses were exposed to experimental reagents in a total volume of 2 ml of control medium for an incubation period of 72-h. Each experimental treatment was performed in triplicate for every horse being tested. At the conclusion of the 72-h exposure period, aliquots of conditioned medium were stored separately at –20°C so that multiple assays could be performed. Tissue culture medium designated for PGE_2 measurement was stored at –20°C with 10 µg/ml of the prostaglandin synthetase inhibitor indomethican.

EXPERIMENTAL DESIGN

In the first part of the study, cartilage explants were exposed to ADA (0.5 to 2 U/ml) to deplete endogenously released adenosine. In addition, control explants were treated with heat-denatured ADA (95°C for 15 min) to verify that the observed effects of ADA exposure were due to enzymatic activity and not to impurities. Finally, explants were exposed to inosine (1 to 100 µM) to evaluate whether products of adenosine metabolism could be responsible for the effects that were observed.

The next part of the study was designed to confirm whether the observed effects of ADA exposure were adenosine-dependent. We first evaluated whether we could limit the effects of ADA by inhibiting other pathways of adenosine degradation. Explants were incubated with the adenosine kinase inhibitor 5′-iodotubercidin (ITU; 0.1 to 1 µM) in the presence of 2 U/ml ADA. Another set of explants was incubated with the ADA-resistant A_{2A} specific receptor agonist N^6-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA; 0.1 to 10 µM) in the presence of 2 U/ml ADA to determine whether the effects of ADA exposure could be reversed by direct adenosine receptor stimulation. Finally, cartilage explants were exposed to one of the following adenosine receptor antagonists to determine whether direct inhibition of adenosine receptor activation could mimic ADA-induced responses: the A_{2A} specific antagonist ZM 241385 (1 µM), the broad antagonist CGS 15943 (1 µM), the broad receptor antagonist caffeine (1 mM), or the broad receptor antagonist theophylline (1 mM).

PROTEOGLYCAN ASSAY

Proteoglycan release was estimated by measuring the concentration of GAG released into the experimental medium using the 1,9-dimethylmethylene blue (DMMB), metachromatic dye assay modified for use in microtiter plates[25]. Shark chondroitin sulfate was used as the standard. Briefly, 10 µl aliquots of medium and standards were pipetted onto a 96-well plate and mixed with 200 µl of 20 µg DMMB/ml (pH 3.3). Optical density was measured at a wavelength of 540 nm with a reference wavelength of 595 nm. All samples and standards were assayed in triplicate and GAG concentration was normalized to µg GAG per mg wet weight.

PGE_2 ASSAY

The concentration of PGE_2 was measured using a commercially available competitive enzyme immunoassay. Briefly, medium was diluted 100 fold in DMEM-F12 and aliquots of diluted sample or standard were added to each well of a microplate that was coated with polyclonal anti-mouse antibody. Subsequently, PGE_2 conjugated to alkaline phosphatase and a monoclonal antibody to PGE_2 were added to each well and the plate was incubated for 2 h. Following incubation, the plate was washed and substrate solution was added to each well. Following a 1-h incubation phosphoric acid was added to each well and optical density was measured at 405 nm with a reference of 570 nm. All samples and standards were assayed in duplicate and PGE_2 production was normalized per mg wet weight.

NO ASSAY

Nitric oxide production by chondrocytes can be accurately estimated by measuring the concentration of nitrite in a conditioned medium sample[27,28]. Nitrite levels were measured using a spectrophotometric assay based on the Greiss reaction[29]. To measure nitrite, 200 µl samples of conditioned medium or standards were mixed with of fresh Greiss reagent centrifuged at 4600 ***g*** for 4 min. After centrifugation, samples were transferred to a 96-well plate and the optical density was measured at 570 nm using a microplate reader. All samples and standards were assayed in triplicate and NO production was normalized per mg wet weight.

MMP-3 ASSAY

Concentration of total MMP-3 in medium was measured using a commercially available solid phase immunoassay. Conditioned medium samples were diluted 10-fold in polypropylene tubes and diluted samples or standards were added with the supplied buffer to wells of a microplate coated with polyclonal anti-MMP-3 antibody. Following a 2-h incubation, the plate was washed and anti-MMP-3 antibody conjugated to horseradish peroxidase was added to each well. After a subsequent 2-h incubation, the plate was washed and substrate solution was added to each well. The microplate was incubated for an additional 30 min before sulfuric acid was added to each well and optical density was measured at 450 nm with a reference of 570 nm. All samples and standards were assayed in duplicate and MMP-3 production was normalized per mg wet weight.

MMP-13 ASSAY

Concentrations of MMP-13 in medium samples were measured using a commercially available solid phase

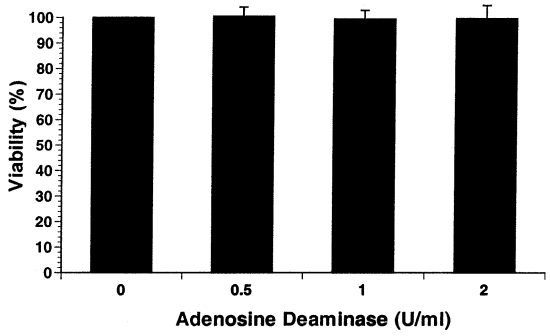

Fig. 2. Effect of ADA exposure on articular chondrocyte viability. Equine chondrocytes were exposed to 0.5 to 2 U/ml ADA for 24 h. ADA did not reduce chondrocyte viability. Values reported represent mean±SEM.

immunoassay. Briefly, aliquots of undiluted sample or standard were added to a microplate coated with anti-MMP-13 antibody and incubated for 2 h. The plate was subsequently washed and anti-MMP-13 antibody conjugated to horseradish peroxidase was added to each well for 1 h before substrate solution was added to each well. After the plate was incubated for another 30 min, sulfuric acid was added to each well and optical density was measured at 450 nm. All samples and standards were assayed in duplicate and MMP-13 production was normalized per mg wet weight.

STATISTICAL ANALYSIS

All values are expressed as mean±SEM. Combined data from all horses were analyzed by use of an ANOVA. When the ANOVA indicated significance, post hoc means comparisons were performed to determine significant differences between individual groups. For experiments in which reagents were added individually, the differences between control measurements and treatment measurements were evaluated using a Dunnett's test ($P<0.05$). Alternatively, for experiments in which mediators were added alone and in combination, comparisons between data pairs were performed by use of the Student *t*-test ($P<0.05$). All analysis was performed using the SAS 8™ statistical software package (SAS Institute, Cary, NC, U.S.A.).

Results

EXPOSURE TO ADA DID NOT SIGNIFICANTLY AFFECT CHONDROCYTE VIABILITY

Chondrocytes in monolayer were exposed to ADA (0.5 to 2 U/ml) in order to determine the effect of ADA on chondrocyte viability. Exposure to ADA did not significantly affect the viability of chondrocytes (Fig. 2).

DEPLETION OF ENDOGENOUS ADENOSINE THROUGH EXPOSURE TO ADA RESULTED IN CARTILAGE MATRIX DEGRADATION, MMP, PGE_2, AND NO RELEASE

Cartilage explants were exposed to ADA (0.5 to 2 U/ml) in order to determine the effects of depleting endogenously produced adenosine on the regulation of cartilage matrix homeostasis. Explants cultured in the presence of ADA released significantly higher levels of GAG into the conditioned medium, indicating enhanced degradation of matrix proteoglycan (Fig. 3A). Similarly, exposure to ADA resulted in a concentration-dependent increase in the synthesis of PGE_2 (Fig. 3B) and NO release (Fig. 3C). Finally, cartilage exposed to ADA produced higher amounts of total MMP-3 (Fig. 3D) and MMP-13 (Fig. 3E). Exposure to heat-denatured ADA did not increase GAG, NO or PGE_2 release when compared to explants cultured in control tissue culture medium alone. Similarly, explants incubated with inosine (1 to 100 μM), a product of adenosine breakdown by ADA, did not increase release of GAG, NO or PGE_2.

AN ADENOSINE KINASE INHIBITOR LIMITED THE EFFECTS OF ADA EXPOSURE

Cartilage explants exposed to ADA (2 U/ml) were co-incubated with the adenosine kinase inhibitor ITU (0.1 to 1 μM) in order to determine whether reducing the enzymatic utilization of adenosine could counteract the effects of ADA exposure. Incubation with ITU (0.2 to 1 μM) significantly decreased GAG (Fig. 4A) and PGE_2 (Fig. 4B) release from ADA-exposed cartilage explants, to the extent that co-addition of 0.5 or 1 μM ITU resulted in GAG and PGE_2 levels that were not significantly higher than release from control tissue. Similarly, incubation with ITU (0.2 to 1 μM) decreased NO production from ADA-exposed explants in a concentration-dependent manner (Fig. 4C) with NO release returning to control levels at 1 μM ITU. In contrast, co-incubation with ADA and ITU at these concentrations did not effectively inhibit ADA-mediated increases in total MMP-3 production (Fig. 4D). Higher concentrations of ITU (0.5 and 1 μM) did, however, reduce ADA-induced MMP-13 to levels that were not significantly different from control explants (Fig. 4E). Exposure to ITU (0.1 to 1 μM) in the absence of ADA did not significantly alter GAG release, PGE_2 synthesis, NO production, total MMP-3 or MMP-13 production when compared to explants cultured in control tissue culture medium alone.

AN A_{2A} ADENOSINE RECEPTOR AGONIST LIMITED THE EFFECTS OF ADA EXPOSURE

Cartilage explants exposed to ADA (2 U/ml) were incubated with the A_{2A} agonist DPMA (0.1 to 10 μM) to determine whether direct adenosine receptor stimulation could reverse the adverse effects of ADA exposure. Incubation with DPMA significantly reduced GAG release (Fig. 5A) and PGE_2 production (Fig. 5B) from cartilage explants stimulated with ADA. Similarly, incubation with DPMA (1 to 10 μM) decreased ADA-induced NO production (Fig. 5C). In the presence of both ADA and DPMA, glycosaminoglycan, NO and PGE_2 levels remained significantly higher than levels produced by control cartilage at all concentrations of DPMA tested. In addition, exposure to DPMA did not alter the ADA-induced increase in total MMP-3 (Fig. 5D) or MMP-13 levels (Fig. 5E). Exposure to DPMA (0.1 to 10 μM) in the absence of ADA did not significantly alter GAG release, PGE_2 synthesis, NO production, total MMP-3 or MMP-13 production relative to levels detected from explants cultured in control tissue culture medium alone.

BLOCKING RECEPTOR ACTIVATION WITH ADENOSINE RECEPTOR ANTAGONISTS ALTERED CARTILAGE MATRIX DEGRADATION AS WELL AS MMP, PGE_2, AND NO RELEASE

Cartilage explants were incubated with different adenosine receptor antagonists in order to evaluate whether

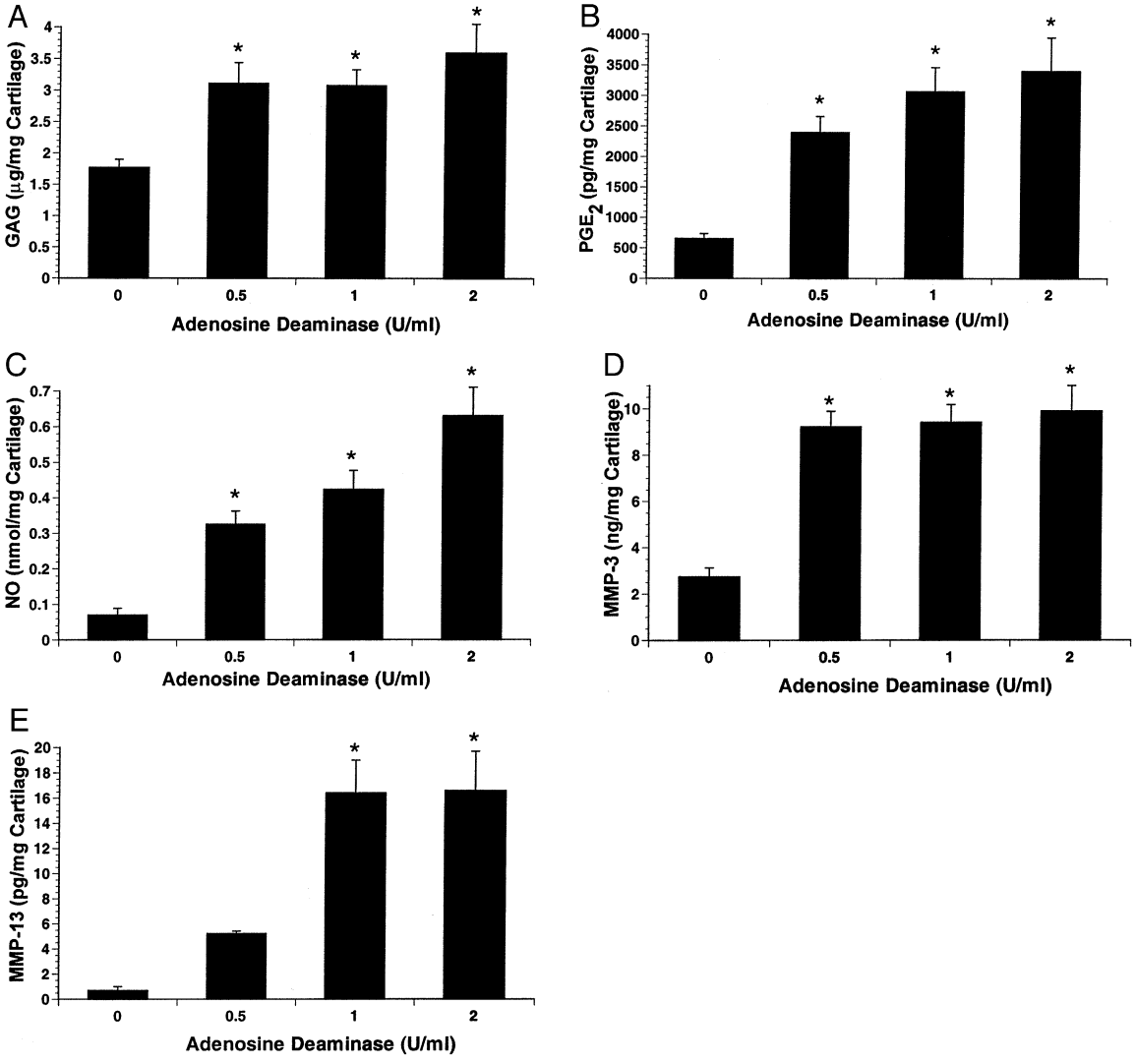

Fig. 3. Effects of ADA-induced adenosine depletion on articular cartilage explant homeostasis. Equine articular cartilage was cultured with 0.5 to 2 U/ml ADA for 72 h. Cartilage exposed to ADA showed a dose-dependent increase in (A) GAG release (N=8), (B) PGE_2 synthesis (N=8), (C) NO production (N=8), (D) total MMP-3 levels (N=6), and (E) MMP-13 (N=3). Values reported represent mean±SEM. *Value is significantly higher than control levels measured in the absence of ADA (P<0.05).

adenosine receptor inhibition would also affect cartilage homeostasis. Exposure to ZM 241385 increased GAG release, total MMP-3, and MMP-13 levels but did not significantly alter PGE_2 or NO production (Table I). Exposure to CGS 15943 increased GAG release and total MMP-3 levels but did not significantly affect PGE_2, NO or MMP-13 production. Incubation with theophylline resulted in an increase in GAG, PGE_2 and NO release but did not significantly change the production of either MMP-3 or MMP-13. In contrast, exposure to caffeine did not significantly alter GAG release, PGE_2 synthesis, NO production, or total MMP-3 and MMP-13 levels compared to explants cultured in control medium alone.

Discussion

The ability of exogenously supplied adenosine to attenuate intraarticular inflammatory responses and limit associated tissue damage in the joint has been documented previously[6,9–11]. The potential significance of this observation is enhanced by recent clinical observations regarding elevated ADA activity measured in patients with naturally occurring arthritic conditions. Increased ADA activity has been documented in the synovial fluid[22,23] and synovial fibroblasts[22] of RA patients and in the serum of patients suffering from RA and systemic lupus erythematosus[22,22,30], and ADA levels correlate well with disease activity in RA[30]. Furthermore, the widely used and highly effective anti-rheumatic drug methotrexate (MTX) has been shown to increase concentrations of extracellular adenosine[31,32] and decrease ADA activity *in vivo*[33]. Based on these studies indicating that a reduction in local concentrations of adenosine may play a significant role in the pathophysiology of naturally occurring joint disease[22,34,35], we theorized that depletion of endogenously released adenosine would be detrimental to cartilage matrix homeostasis in the absence of other mediators of disease.

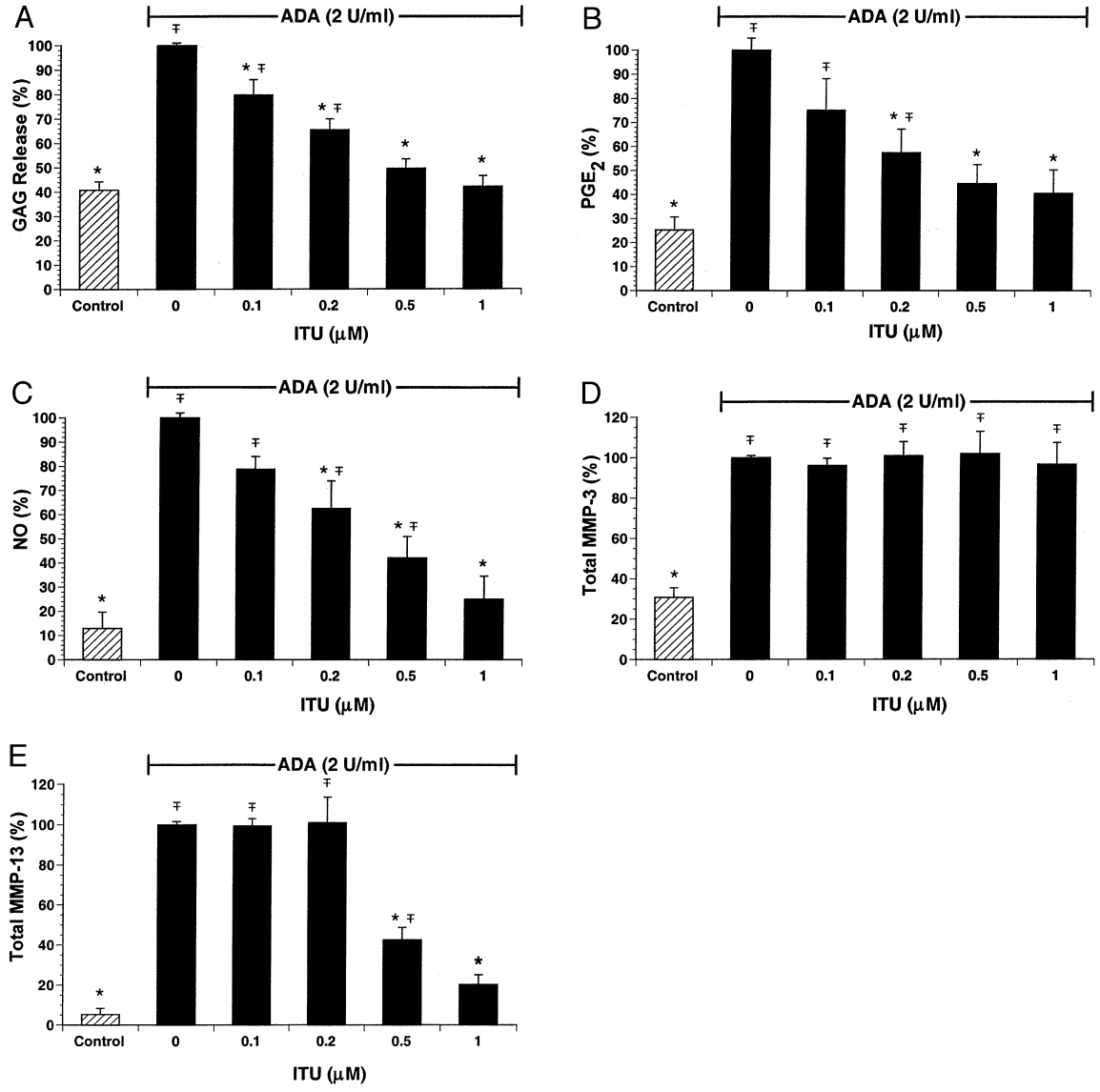

Fig. 4. Effects of adenosine kinase inhibition on ADA-exposed cartilage explants. Equine articular cartilage explants were cultured with 2 U/ml ADA and exposed to 0.1 to 1 μM of the adenosine kinase inhibitor ITU for 72 h. Cartilage exposed to ITU showed a dose-dependent decrease in ADA-mediated (A) GAG release (N=5), (B) PGE_2 synthesis (N=5), and (C) NO production (N=5). Incubation with ITU did not significantly reduce (D) total MMP-3 levels (N=4) but did inhibit ADA-induced (E) MMP-13 production (N=3). Values reported represent mean±SEM. T Value is significantly higher than control levels measured in the absence of ADA (P<0.05). *Value is significantly lower than 2 U/ml ADA alone (P<0.05).

The two major enzymatic pathways that utilize adenosine are phosphorylation by adenosine kinase and deamination by ADA[19]. In this study ADA activity was used to deplete adenosine levels to test our hypothesis. We selected ADA because it has been shown to exist in an extracellular form *in vivo*[20] while adenosine kinase is primarily active in the cytosol[36]. Several isoenzymes of ADA, both free and bound to CD26, with differing molecular weights, kinetic properties and tissue distributions, have been described[22,37]. Adenosine deaminase 1 was chosen for this study because it has a higher affinity for adenosine and a higher catalytic activity with deoxyadenosine[22,38]. In addition, in normal and disease states where elevated ADA activity has been detected, the ADA 1 isozyme has predominated in human tissues and cells[19,20,22].

The concentrations of ADA utilized in this study were higher those measured in the serum (0.0393 U/ml)[30] or synovial fluid (0.0144 U/ml)[22] of RA patients or in serum samples from normal healthy controls (0.0157 U/ml)[30]. However, viability-testing results reported here show that the concentrations of ADA used in this study do not adversely affect chondrocyte viability. Pilot studies performed using up to 8 U/ml ADA indicated that concentration-dependent responses were observed in the range between 0.5 and 2 U/ml ADA while above 2 U/ml cartilage responses plateaued. *In vivo*, cells have the ability to continually synthesize and replace ADA as the protein becomes inactivated, but in this experimental model ADA was added to the culture system only once, at the initiation of experiments. The upper limit of the

Table I

Effects of adenosine receptor antagonists on articular cartilage explants. Equine articular cartilage was cultured for 72 h alone (control) or with one of the following adenosine receptor antagonists: 1 μM of the A_{2A} specific antagonist ZM 241385, 1 μM of the broad antagonist CGS 15943, 1 mM of the broad antagonist theophylline or 1 mM of the broad antagonist caffeine. Cartilage incubated with ZM 241385 increased GAG release and total MMP-3 and MMP-13 levels while exposure to CGS 15943 increased MMP-3 alone. Theophylline exposure caused an increase in GAG release, PGE_2 synthesis and NO production. Values reported represent mean±SEM

Sample	(*N*=4) GAG μg/mg Cart	(*N*=4) PGE_2 pg/mg Cart	(*N*=4) NO nmol/mg Cart	(*N*=3) MMP-3 ng/mg Cart	(*N*=3) MMP-13 pg/mg Cart
Control	2.39±0.29	417±119	0.83±0.43	2.51±0.56	0.67±0.27
1 μM ZM 241385	3.70±0.50*	380±104	0.56±0.26	4.83±0.28*	1.73±0.07*
1 μM CGS 15943	3.46±0.07	364±113	0±0	4.56±0.35*	0.3±0.17
1 mM Theophylline	3.67±0.35*	1761±381*	2.2±0.22*	2.78±0.71	0.53±0.39
1 mM Caffeine	2.46±0.28	464±135	0±0	2.73±0.51	0.73±0.03

*Value is significantly higher than control levels measured in the absence of ADA ($P<0.05$).

concentration-dependent range (2 U/ml) was selected for use in these experiments to ensure enzymatic degradation of endogenous adenosine for the entire 72-h incubation period.

In designing this study, we presumed that cartilage responses to ADA exposure would be due to decreased concentrations of extracellular adenosine. However, we did recognize that ADA supplementation could affect explant homeostasis through other mechanisms, including exposure to products of adenosine degradation. Cartilage exposed to inosine, the product of adenosine breakdown by ADA, did not increase matrix degradation or second messenger release, suggesting that inosine did not play an important role in the observed effects of ADA exposure in this study. Cartilage explants were also exposed to heat-denatured ADA to test for potential contamination of the enzyme preparation with endotoxin. Heat treatment to denature ADA under conditions under which lipopolysaccharide was not expected to be adversely affected[39] abolished ADA-induced release of GAG, NO and PGE_2, demonstrating that endotoxin contamination was not responsible for the effects of ADA exposure observed in this study.

This study included several additional experiments designed to confirm that ADA was acting via adenosine depletion. Previous work demonstrated that ITU causes chondrocytes to accumulate high concentrations of extracellular adenosine[12]. The ability of ITU to increase extracellular adenosine is most likely due to enzyme kinase inhibition, although ITU has been demonstrated to inhibit nucleoside transport with a K_i higher than the concentrations of ITU utilized in this study[40]. In this study, co-exposure of cartilage to ITU inhibited ADA-induced effects in a concentration-dependent manner, indicating that ITU may be directly antagonizing the ability of ADA to reduce the concentration of extracellular adenosine. Similar results were obtained following co-incubation with ADA and the A_{2A} specific agonist DPMA. Receptor agonists including DPMA, signal through cell surface receptors, mimicking the effects of extracellular adenosine[7] and chondrocytes have been shown to possess transcripts for P1 receptors[41]. The ability of DPMA to modulate cartilage responses to ADA exposure suggests that in addition to promoting anti-inflammatory responses[7,8,10,42,43], adenosine receptors, in particular A_{2A}, may also be important in delivering signals that instruct chondrocytes to maintain cartilage matrix. Together, these results provide good evidence that the observed effects of ADA exposure were mediated by reduced adenosine concentrations.

Adenosine deaminase can bind to A_1 and the low affinity A_{2B} adenosine receptors and increase receptor ligand affinity[44–47], which suggests an alternative role for ADA in this study. However, the significance of this phenomenon for interpretation of the current study is difficult to assess. The original experiments demonstrating ADA receptor binding were performed using a cell line with admittedly high expression levels of A_1 receptor[44,45]. In addition, experiments demonstrating an increased ligand receptor affinity in the presence of ADA used a synthetic receptor agonist. Chondrocytes exposed to ADA alone in this study would only have adenosine available as an agonist and an evaluation of the ability of adenosine to bind cell surface receptors before being degraded by ADA is beyond the scope of this study. It is unlikely that the effects of ADA exposure observed in this study are due to ADA-mediated increases in A_{2B} binding as previous work has demonstrated that Gs stimulation and increased cAMP are linked to decreased NO release[7], which is opposed to results reported in this study. Finally, the A_1 and A_2 subclasses of adenosine receptors produce antagonizing effects on adenylyl cyclase and cAMP production and the net effect of ADA-enhanced receptor binding would be dependent upon which receptor was present in greater numbers.

The last section of the study reported here was designed to reproduce ADA-like responses indirectly, without decreasing extracellular adenosine by using adenosine receptor antagonists to block receptor stimulation by endogenous adenosine. The conclusion that A_{2A} receptors play a role in regulating matrix homeostasis was supported by the ability of the A_{2A} specific antagonist ZM 241385 to mimic the effects of ADA exposure and increase GAG release and MMP levels. Although exposure to the A_{2A} specific antagonist ZM 241385 increased cartilage catabolic activity, not all of the receptor antagonists used in this study caused measurable responses. These results are similar to those reported in another study is which exposure to caffeine or theophylline alone had no effect in an *in vivo* rat adjuvant arthritis model of RA[34]. Furthermore, unstimulated chondrocytes accumulate only low levels of extracellular adenosine[13] and it is possible that lower affinity antagonists may not be able to block enough of the low, tonic receptor stimulation to see changes in cartilage homeostasis. In addition to blocking adenosine receptors, theophylline is known to be a phosphodiesterase inhibitor[48] and the observed changes in cartilage metabolism may be due to inadvertent effects of the reagent.

Chondrocytes have been shown to release adenosine in response to inflammatory stimuli[13], and endogenous adenosine may provide a negative feedback loop to limit cartilage damage after the initiation of inflammatory responses[14]. This suggests that enzymes other than ADA that affect the concentration of extracellular adenosine may

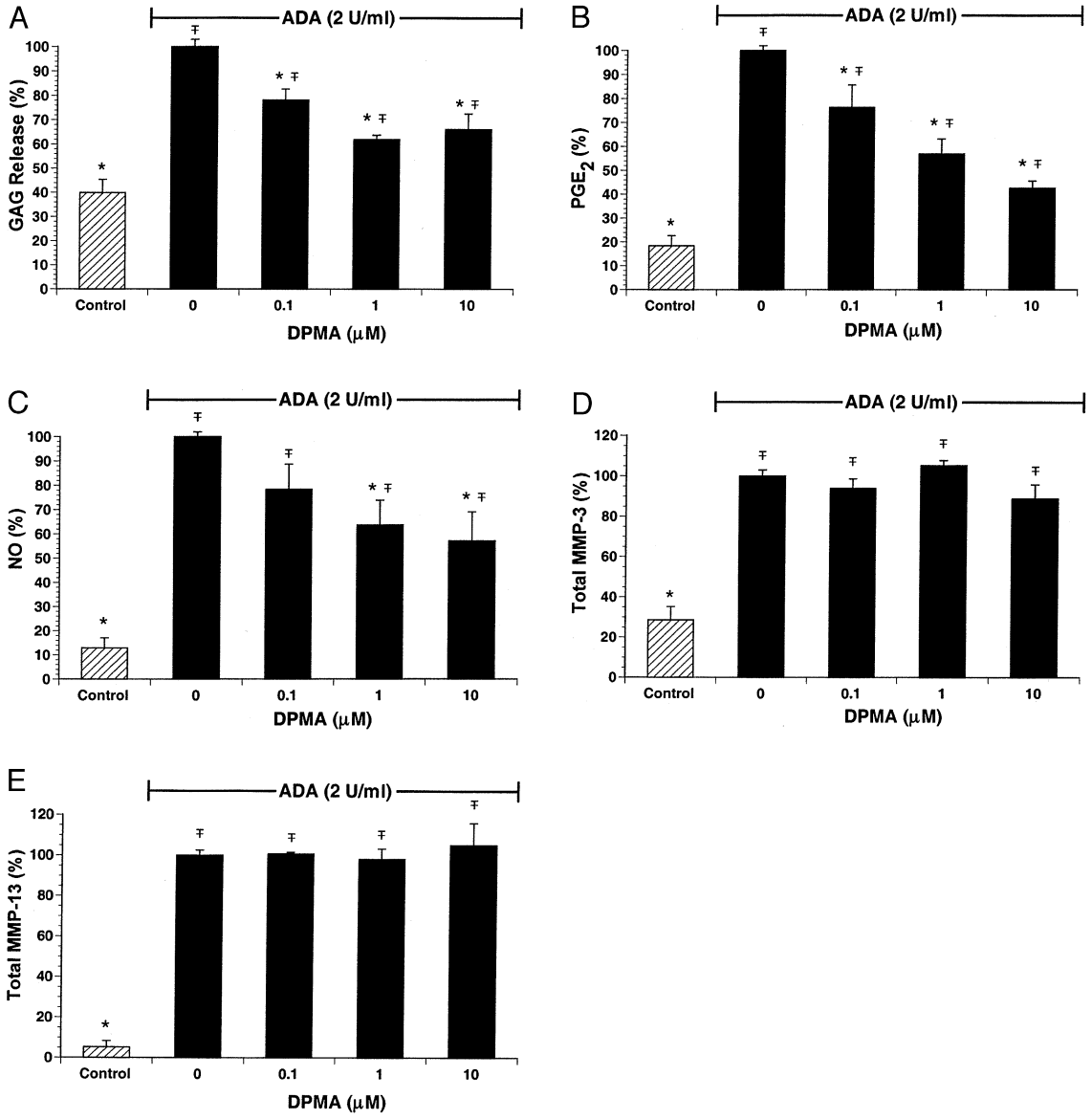

Fig. 5. Effects of selective A_{2A} adenosine receptor stimulation on ADA-exposed cartilage explants. Equine articular cartilage was cultured with 2 U/ml ADA and exposed to 0.1 to 10 μM of the A_{2A} receptor agonist DPMA for 72 h. Incubation with DPMA inhibited ADA-induced (A) GAG release (N=5), (B) PGE_2 synthesis (N=5), and (C) NO production (N=5) but did not decrease (D) total MMP-3 levels (N=4), or (E) MMP-13 (N=3). Values reported represent mean±SEM. T Value is significantly higher than control levels measured in the absence of ADA (P<0.05). *Value is significantly lower than 2 U/ml ADA alone (P<0.05).

also be capable of modulating cartilage homeostasis. In support of this, increased activity of the key[49] adenosine generating enzyme 5′ nucleotidase (5NT) has been found in diseased cartilage[50], suggesting that 5NT may be up-regulated in response to disease. Further investigation into the relationship between 5NT, extracellular adenosine levels and cartilage homeostasis is certainly warranted. In the current study GAG release was measured as an indicator of proteoglycan degradation, and production of MMP-3 (stromelysin-1) and MMP-13 (collagenase-3) were quantified as markers of matrix degradative activity[51]. Stromelysin-1, which was increased by exposure to ADA, has been found in synovial fluid samples from patients with a variety of pathologic conditions including RA[52] and has also been show to activate a variety of other catabolic enzymes[53]. Collagenase-3, which was also increased with exposure to ADA, is thought to be a key mediator in OA associated with chondrocyte mediated cartilage destruction[54], and may be the primary collagenase responsible for matrix degradation in OA cartilage[55].

Although ADA and an A_{2A}-selective receptor antagonist both initiated an increase in MMP production, the control of MMP expression may not be entirely adenosine-dependent. The A_{2A} agonist DPMA was unable to inhibit ADA-induced MMP-3 or MMP-13 expression. Similarly, incubation with ITU did not inhibit MMP-3 production in response to ADA. The inability of both receptor-dependent and receptor-independent adenosine modulation to inhibit ADA-induced increases in MMP-3 levels suggests that, once production is initiated, the mechanism

of down-regulating these enzymes may be complex and should be the focus of further study.

This study reveals that endogenously released adenosine can regulate chondrocyte production of matrix degrading enzymes and matrix loss. In a previous study measuring adenosine release from articular chondrocytes cultured in monolayer, no adenosine was detected in conditioned medium from unstimulated cells[13]. However, it is likely that adenosine levels below the sensitivity threshold of the assay (<100 nM) were present in the medium and that those levels would be sufficient to result in intracellular signaling. In addition, higher levels of adenosine could be expected to be present at the cell surface. Alternatively, considering the extremely short half-life of adenosine in biological systems[14–16], higher levels of adenosine may have been release by unstimulated cells in that study, but have been naturally degraded to levels below the detection threshold prior to completion of the high performance liquid chromatography assay.

Studies of naturally occurring joint pathology have suggested that local adenosine levels are important in disease and *in vitro* work has indicated that adenosine modulates chondrocyte responses to inflammatory stimuli. The purpose of this study was to investigate the potential of endogenous adenosine as an autocrine and paracrine regulator of articular chondrocytes. The long-term goal of adenosine research is to develop an agent that can limit joint damage that occurs with injury or disease. Release of adenosine from chondrocytes appears to play a role in the cellular response to tissue damage in arthritic conditions, and pharmacologic modulation of these pathways in joints may have therapeutic potential. This study demonstrates that endogenous adenosine affects cartilage matrix integrity in the absence of inflammatory stimuli and strengthens the hypothesis that adenosine is a powerful signaling agent in the joint.

References

1. Cronstein BN, Kramer SB, Weissmann G, Hirschhorn R. Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. J Exp Med 1983;158:1160–77.
2. Le Vraux V, Chen YL, Masson I, De Sousa M, Giroud JP, Florentin I, *et al.* Inhibition of human monocyte TNF production by adenosine receptor agonists. Life Sci 1993;52:1917–24.
3. Poon A, Sawynok J. Antinociceptive and anti-inflammatory properties of an adenosine kinase inhibitor and an adenosine deaminase inhibitor. Eu J Pharmacol 1999;384:123–38.
4. Siegmund B, Rieder F, Albrich S, Wolf K, Bidlingmaier C, Firestein GS, *et al.* Adenosine kinase inhibitor GP515 improves experimental colitis in mice. J Pharmacol Exp Ther 2001;296:99–105.
5. McGaraughty S, Chu KL, Wismer CT, Mikusa J, Zhu CZ, Cowart M, *et al.* Effects of A-134974, a novel adenosine kinase inhibitor, on carrageenan-induced inflammatory hyperalgesia and locomotor activity in rats: evaluation of the sites of action. J Pharmacol Exp Ther 2001;296:501–9.
6. Boyle DL, Sajjadi FG, Firestein GS. Inhibition of synoviocytes collagenase gene expression by adenosine receptor stimulation. Arthritis Rheum 1996;39:923–30.
7. Tesch AM, MacDonald MH, Kollias-Baker C, Benton HP. Chondrocytes respond to adenosine via A2 receptors and activity is potentiated by an adenosine deaminase inhibitor and a phosphodiesterase inhibitor. Osteoarthritis Cart 2002;10:34–43.
8. Benton HP, MacDonald MH, Tesch AM. Effects of adenosine on bacterial lipopolysaccharide- and interleukin 1-induced nitric oxide release from equine articular chondrocytes. Am J Vet Res 2002;63: 204–10.
9. Green PG, Basbaum AI, Helms C, Levine JD. Purinergic regulation of bradykinin-induced plasma extravasation and adjuvant-induced arthritis in the rat. Neurobiol 1991;88:4162–5.
10. Szabo C, Scott GS, Virag L, Egnaczyk G, Salzman AL, Shanley TP, *et al.* Suppression of macrophage inflammatory protein (MIP)-1alpha production and collagen-induced arthritis by adenosine receptor agonists. Br J Pharmacol 1998;125:379–87.
11. Picher M, Graff RD, Lee GM. Extracellular nucleotide metabolism and signaling in the pathophysiology of articular cartilage. Arthritis Rheum 2003;48:2722–36.
12. Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. Structure and function of adenosine receptors and their genes. Arch Pharmacol 2000; 362:364–74.
13. Tesch AM, MacDonald MH, Kollias-Baker C, Benton HP. Effects of an adenosine kinase inhibitor and an adenosine deaminase inhibitor on accumulation of extracellular adenosine by equine articular chondrocytes. Am J Vet Res 2002;63:1512–9.
14. Schaeffer HJ, Schwender CF. Enzyme inhibitors. 26. Bridging hydrophobic and hydrophilic regions on adenosine deaminase with some 9-(2-hydroxy-3-alkyl)adenines. J Med Chem 1974;17:6–8.
15. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. Am J Physiol 1989;256:C799–806.
16. Dawicki DD, Agarwal KC, Parks RE. Adenosine metabolism in human whole blood: effects of nucleoside transport inhibitors and phosphate concentration. Biochem Pharmacol 1988;37:621–6.
17. Ugarkar BG, Castellino AJ, DaRe JM, Kopcho JJ, Wiesner JB, Schanzer JM, *et al.* Adenosine kinase inhibitors. 2. Synthesis, enzyme inhibition, and anti-seizure activity of diaryltubercidin analogues. J Med Chem 2000;43:2894–905.
18. Cristalli G, Costanzi S, Lambertucci C, Lupidi G, Vittori S, Volpini R, *et al.* Adenosine deaminase: functional implications and different classes of inhibitors. Med Res Rev 2001;21:105–28.
19. Ungerer JP, Oosthuizen HM, Bissbort SH, Vermaak WJ. Serum adenosine deaminase: isoenzymes and diagnostic application. Clin Chem 1992;38:1322–6.
20. Ratech H, Hirschhorn R. Serum adenosine deaminase in normals and in a patient with adenosine deaminase deficient-severe combined immunodeficiency. Clin Chim Acta 1981;115:341–7.
21. Hitoglou S, Hatzistilianou M, Gougoustamou D, Athanassiadou F, Kotsis A, Catriu D. Adenosine deaminase activity and its isoenzyme pattern in patients with juvenile rheumatoid arthritis and systemic lupus erythematosus. Clin Rheumatol 2001; 20:411–6.
22. Nakamachi Y, Koshiba M, Nakazawa T, Hatachi S, Saura R, Kurosaka M, *et al.* Specific increase in

enzymatic activity of adenosine deaminase 1 in rheumatoid synovial fibroblasts. Arthritis Rheum 2003; 48:668–74.
23. Iwaki-Egawa S, Watanabe Y, Matsuno H. Correlations between matrix-metalloproteinase-9 and adenosine deaminase isoenzymes in synovial fluid from patients with rheumatoid arthritis. J Rheumatol 2001; 28:485–9.
24. Deussen A, Stappert M, Schafer S, Kelm M. Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. Circulation 1999;99:2041–7.
25. MacDonald MH, Tesch AM, Benton HP, Willits NH. Characterization of age- and location-associated variations in the composition of articular cartilage from the equine metacarpophalangeal joint. J Equine Vet Sci 2002;22:25–32.
26. Plumb JA, Milroy R, Kaye SB. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res 1989;49: 4435–40.
27. Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, *et al.* Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. J Immunol 1991;147: 3915–20.
28. Stefanovic-Racic M, Morales TI, Taskiran D, McIntyre LA, Evans CH. The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. J Immunol 1996;156:1213–20.
29. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
30. Sari RA, Taysi S, Yalimaz O, Bakan N. Correlation of serum levels of adenosine deaminase activity and its isoenzymes with disease activity in rheumatoid arthritis. Clin Exp Rheumatol 2003;21:87–90.
31. Morabito L, Montesinos MC, Schreibman DM, Balter L, Thompson LF, Resta R, *et al.* Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. J Clinical Invest 1998;101:295–300.
32. Chan ES, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis Res 2002; 4:266–73.
33. van Ede AE, Laan RFJM, De Abreu RA, Stegeman ABJ, van de Putte LBA. Purine enzymes in patients with rheumatoid arthritis treated with methotrexate. Ann Rheum Dis 2002;61:1060–4.
34. Montesinos MC, Yap JS, Desai A, Psadas I, McCrary CT, Cronstein BN. Reversal of the antiinflammatory effects of methotrexate by the nonselective receptor antagonists theophylline and caffeine. Arthritis Rheum 2000;43:656–63.
35. Cronstein BN. The antirheumatic agents suphasalazine and methotrexate share and anti-inflammatory mechanism. Br J Rheumatol 1995;2(34 Suppl):30–2.
36. Yamada Y, Goto H, Ogasawara N. Purification and properties of adenosine kinase from rat brain. Biochem Biophys Acta 1980;616:199–207.
37. Hirschhorn R, Ratech H. Isozymes of adenosine deaminase. Isozymes Curr Top Biol Med Res 1980; 4:131–57.
38. Ratech H, Thorbecke GJ, Meredith G, Hirschhorn R. Comparison and possible homology of isozymes of adenosine deaminase in Aves and humans. Enzyme 1981;26:74–84.
39. Okamura Y, Watari M, Jerud ES, Young DW, Ishizaka ST, Rode J, *et al.* The extra domain A of fibronectin activates toll-like receptor 4. J Biol Chem 2001; 276:10229–33.
40. Parkinson FE, Geiger JD. Effects of iodotubercidin on adenosine kinase activity and nucleoside transport in DDT1 MF-2 smooth muscle cells. J Pharmacol Exp Ther 1996;277:1397–401.
41. Koolpe M, Pearson D, Benton HP. Expression of both P1 and P2 purine receptor genes by human articular chondrocytes and profile of ligand-mediated prostaglandin E2 release. Arthritis Rheum 1999;42:258–67.
42. Sullivan GW, Linden J, Buster BL, Scheld WM. Neutrophil A2A adenosine receptor inhibits inflammation in a rat model of meningitis: synergy with the type IV phosphodiesterase inhibitor, rolipram. J Infect Dis 1999;180:1550–60.
43. Bouma MG, Stad RK, van den Wildenberg AJM, Buurman WA. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. J Immunol 1994;153:4159–68.
44. Ciruela F, Saura C, Canela E, Mallol J, Lluis C, Franco R. Adenosine deaminase affects ligand-induced signaling by interacting with cell surface adenosine receptors. FEBS Lett 1996;380:219–23.
45. Saura C, Mallol J, Canela E, Lluis C, Franco R. Adenosine deaminase and A1 adenosine receptors internalize together following agonist-induced receptor desensitization. J Biol Chem 1998;273:17610–7.
46. Herrera C, Casado F, Ciruela F, Schofield P, Mallol J, Lluis C, *et al.* Adenosine A2B receptors behave as an alternative anchoring protein for cell surface adenosine deaminase in lymphocytes and cultured cells. Mol Pharmacol 2001;59:127–34.
47. Torvinen M, Gines S, Hillion J, Latini S, Canals M, Ciruela F, *et al.* Interactions among adenosine deaminase, adenosine A1 receptors and dopamine D1 receptors in stably cotransfected fibroblast cells and neurons. Neurosci 2002;113:709–19.
48. Wang CH, Lin HC, Lin CH, Yu CT, Liu SL, Huang KH, *et al.* Effect of theophylline and specific phosphodiesterase IV inhibition on proliferation and apoptosis of progenitor cells in bronchial asthma. Br J Pharmacol 2003;138:1147–55.
49. Morabito L, Montesinos MC, Schreibman DM, Balter L, Thompson LF, Resta R, *et al.* Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. J Clin Invest 1998;101:295–300.
50. Tenenbaum J, Muniz O, Schumacher HR, Good AE, Howell DS. Comparison of phosphohydrolase activities from articular cartilage in calcium pyrophosphate deposition disease and primary osteoarthritis. Arthritis Rheum 1981;24:492–500.
51. Kozaci LD, Brown CJ, Adcocks C, Galloway A, Hollander AP, Buttle DJ. Stromelysin 1, neutrophil collagenase, and collagenase 3 do not play major roles in a model of chondrocyte mediated cartilage breakdown. J Clin Pathol Mol Pathol 1998;51:282–6.
52. McCachren SS. Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. Arthritis Rheum 1991;34:1085–92.

53. Murphy G, Knauper V, Atkinson S, Butler G, English W, Hutton M, *et al.* Matrix metalloproteinases in arthritic disease. Arthritis Res 2002;4:S39–43.
54. Tetlow LC, Woolley DE. Comparitive immunolocalization studies of collagenase 1 and collagenase 3 production in the rheumatoid lesion, and by human chondrocytes and synoviocytes. Br J Rheumatol 1998;37:64–70.
55. Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum 2002;46:2648–57.